THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 22, Issue of August 5, pp. 16578-16583, 1993
Printed in U.S.A.

A Kinase-related Protein Stabilizes Unphosphorylated Smooth Muscle Myosin Minifilaments in the Presence of ATP*

(Received for publication, November 16, 1992, and in revised form, March 30, 1993)

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An apparent paradox in smooth muscle biology is the ability of unphosphorylated myosin to maintain a filamentous structure in the presence of ATP *in vivo*, whereas unphosphorylated myosin filaments are depolymerized *in vitro* in the presence of ATP. This suggests that additional uncharacterized factors are required for the stabilization of myosin filaments in the presence of ATP. We report here that an abundant smooth muscle protein forms sedimentable complexes with unphosphorylated smooth muscle myosin, partially reverses the depolymerizing effect of ATP on unphosphorylated myosin, and promotes the assembly of minifilaments as revealed by electron microscopy. This protein is called kinase-related protein (KRP) because it is derived from a gene within the gene for myosin light chain kinase (MLCK) and has an amino acid sequence identical to the carboxyl-terminal domain of MLCK. Consistent with the results with purified KRP, deletion of the KRP domain within MLCK results in a diminished ability of MLCK to interact with unphosphorylated myosin. KRP binds to the heavy meromyosin fragment of myosin but not to myosin rod or fragments lacking the hinge region and light chains. Altogether, these results suggest that KRP may play a critical role in stabilizing unphosphorylated myosin filaments and that the KRP domain of MLCK may be important for subcellular targeting to filaments.

One of the key proteins involved in smooth muscle contraction is myosin. Myosin phosphorylation on the light chain subunit by the calcium- and calmodulin (CaM)[1]-dependent enzyme myosin light chain kinase (MLCK) is essential for calcium-mediated smooth muscle contraction, and relaxation is usually accompanied by myosin dephosphorylation (for a review, see Murphy (1989) and Ruegg (1986)). Biochemical data indicate that filamentous smooth muscle myosin with unphosphorylated light chains is depolymerized to a monomeric folded form *in vitro* by MgATP (Craig *et al.*, 1983; Onishi *et al.*, 1982; Suzuki *et al.*, 1978; Trybus *et al.*, 1982). This, however, appears to contradict electron microscopy results, which demonstrate the presence of myosin filaments in intact smooth muscle even when the muscle is relaxed and light chain phosphorylation does not exceed 5% (Somlyo *et al.*, 1981; Gillis *et al.*, 1988). Therefore, other unknown factors must be present in smooth muscle in order to account for the myosin filament stability *in vivo* under conditions where the level of myosin light chain phosphorylation is low and ATP is present.

We report here evidence that the smooth muscle kinase-related protein, KRP (Collinge *et al.*, 1992; Shattuck *et al.*, 1988), has the potential of being one of the unknown factors required for the stability of unphosphorylated myosin filaments. KRP is an abundant smooth muscle protein that has an amino acid sequence identical to the carboxyl-terminal domain of MLCK (Collinge *et al.*, 1992; Shoemaker *et al.*, 1990). KRP is encoded by a 2.7-kb mRNA, whereas MLCK is encoded in the same tissue by a 5.5-kb mRNA (Collinge *et al.*, 1992). The complete DNA sequence of the chicken KRP transcription unit has been elucidated, the site of transcription initiation mapped by primer extension and nuclease protection, the genomic relationship between the KRP gene and the MLCK gene determined, and the purified KRP characterized (Collinge *et al.*, 1992). These previous studies (Collinge *et al.*, 1992; Shoemaker *et al.*, 1990) demonstrated that the unusual structural relatedness of KRP and MLCK is due to a novel gene within a gene relationship in which the KRP gene promoter and site of initiation are within an MLCK intron, but the coding region of the KRP mRNA is produced by the splicing of a set of three exons that are also used in the production of the larger mRNA encoding MLCK. This results in a KRP mRNA that has a 5′-noncoding sequence derived from MLCK gene intron and exon sequences and a KRP translation initiation codon that is an internal methionine codon for MLCK. The use of the common reading frame in the two mRNA species is the molecular mechanism by which KRP has an amino acid sequence identical to a domain of MLCK. KRP does not encode CaM regulatory or protein kinase activity (Collinge *et al.*, 1992), consistent with the segmental organization of function within MLCK (Shoemaker *et al.*, 1990). Thus, a function for KRP has not been reported yet.

In this study, we demonstrate that chicken gizzard KRP binds to unphosphorylated gizzard myosin and is capable of reversing the depolymerizing effect of MgATP on unphosphorylated myosin filaments. The concentration of KRP and KRP mRNA in chicken gizzard is greater than that of MLCK

* These studies were supported in part by National Institutes of Health Grants GM30861 and HL47070. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CaM, calmodulin; kb, kilobase(s); MLCK, myosin light chain kinase; KRP, kinase-related protein; HMM, heavy meromyosin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; EM, electron microscopy; MOPS, 4-morpholinepropanesulfonic acid.

and is comparable to that of myosin. Low molecular weight proteins with similarity to the carboxyl-terminal domain of MLCK have also been detected in turkey gizzard (Ito *et al.*, 1989) and rabbit smooth muscle tissues (Gallagher and Herring, 1991) and have been given the name telokin because of this similarity. Altogether, these previous reports (Collinge *et al.*, 1992; Gallagher and Herring, 1991; Ito *et al.*, 1989) indicate that abundant smooth muscle kinase-related proteins are produced in multiple vertebrate species, although their mechanism of production has yet to be shown to be similar. Therefore, we propose that this class of proteins be referred to as myofilins based on the myosin filament stabilization function demonstrated here.

MATERIALS AND METHODS

Proteins—KRP used in these studies was purified from freshly frozen chicken gizzards by using one of the multiple previously described methods (Collinge *et al.*, 1992; Ito *et al.*, 1989; Shattuck *et al.*, 1988; Van Eldik and Watterson, 1979), with no detectable differences in amino acid composition, gel mobility, or function among the resultant purified protein. Phosphorylated turkey gizzard myosin and unphosphorylated turkey gizzard myosin containing less than 5% phosphorylated myosin were prepared as described by Sellers *et al.* (1981) and Sellers and Pato (1984). The HMM (Sellers, 1985), S1 (Ikebe and Hartshorne, 1984), S2 (Sellers and Harvey, 1984), and myosin rod (Sellers and Harvey, 1984) fractions of gizzard myosin were prepared as previously described. Rabbit skeletal muscle myosin was isolated as described by Margossian and Lowey (1982). Actins were purified as previously described (Eisenberg and Kielley, 1974; Pardee and Spudich, 1982). Gizzard MLCK was purified following the general method described by Adelstein and Klee (1982) and further purified by ion exchange chromatography on a Mono Q column essentially as described (Shoemaker *et al.*, 1990; Fasolo *et al.*, 1991). KRP stock solutions were quantitated based upon amino acid analysis of acid hydrolysates (Schaefer *et al.*, 1987; Collinge *et al.*, 1992); from a solution of known concentration, the extinction coefficient ($\epsilon_{280\,nm}^{mg/ml}$) of KRP was calculated to be 0.78. Other proteins were quantitated by standard protein assays (Lowry *et al.*, 1951), amino acid analysis (CaM and MLCK) or published (Sellers and Pato, 1984) extinction coefficients; a molecular weight of 500,000 was used for myosin.

Sedimentation Assays—Proteins were dialyzed in buffer A (10 mM MOPS, pH 7.0, 50 mM NaCl, 10 mM β-mercaptoethanol (or 1 mM DTT), 1 mM $MgCl_2$, 1 mM EGTA) containing, where indicated, 1 mM ATP and 100 mM NaCl. Myosin filaments were prepared by dialyzing solubilized myosin (0.5 M NaCl or 0.6 M KCl) into buffer A. Solutions of KRP and MLCK were dialyzed against buffer A when necessary to lower salt concentrations to 50 mM.

Samples of KRP were tested for the presence of myosin phosphorylation activity by incubation of gizzard myosin (5 μM) and KRP (10 μM) in 50 mM HEPES buffer, pH 7.5, containing 1 mM [γ-^{32}P] ATP (70 cpm/pmol), 5 mM $MgCl_2$, 0. 1 mM $CaCl_2$, and 1 mM DTT for 30 min at 25 °C. The reaction was terminated by addition of 4 × SDS sample buffer (Laemmli, 1970) and then subjected to SDS-PAGE. Autoradiography of the gel indicated no detectable phosphorylation of myosin. Preparations of myosin were readily phosphorylated by the addition of MLCK and calmodulin as described previously (Sellers *et al.*, 1981).

Myosin with or without added proteins was incubated in buffer A (final volume 100 μl) for 20 min at 20–25 °C, and centrifuged at 85,000 × *g* for 30 min (Beckman TL100 centrifuge with a TL100.2 rotor). Monomeric myosin, KRP, or MLCK solutions did not pellet under these conditions. The pellets were resuspended in 100 μl of water each, and samples were prepared for electrophoresis by adding 33 μl of 4 × Laemmli sample buffer (Laemmli, 1970) to the supernatant and pellets. Solutions were vortexed to resuspend proteins and then boiled for 3 min before electrophoresis was performed.

In the case of HMM and S1, 5 μM HMM or S1 were mixed with 20 μM actin in Buffer A. The KRP concentration was 5 μM. Sedimentation was 436,000 × *g* for 10 min in a Beckman TL100 centrifuge using a TL100.2 rotor. Pellets were rinsed once with buffer A and resuspended in SDS sample buffer. Electrophoresis was on a 12.5% (w/v) polyacrylamide gel with SDS.

For competition experiments with S2, HMM (5 μM), actin (20 μM), and KRP (5 μM) were mixed with 25 μM S2. Following sedimentation as above, the supernatant and pellets were analyzed by SDS-PAGE on 12.5% polyacrylamide gels.

Phosphorylation Assays—In sedimentation experiments where MLCK activity was measured from the supernatant, sample tubes were pretreated with solutions of 5 mg/ml bovine serum albumin in buffer A for 30 min at 20–25 °C, and bovine serum albumin was included in the incubation buffer at a concentration of 0.5 mg/ml as described by Sellers and Pato (1984). The sedimentation experiment was done as above except that the MLCK concentration was 0.05 μM and myosin at 2.5 or 5.0 μM. After pelleting at 4 °C, the samples were placed on ice, supernatant was removed, and MLCK activity was measured from an aliquot of the supernatant using a synthetic peptide phosphorylation assay as described previously (Haiech *et al.*, 1991).

HMM phosphorylation by MLCK was done by the method described by Sellers *et al.* (1981), except that the final CaM concentration was 2 μM, and final MLCK concentration 0.0005 μM. Initial rates were estimated from aliquots removed from the reaction at 30-s intervals from 0 to 2 min. Incorporation of phosphate into the HMM was linear in this time period.

Gel Electrophoresis and Densitometry—Samples were electrophoresed in 12.5% acrylamide minislab gels; gels were stained with Coomassie Blue R-250 dye, destained, and scanned on a densitometer (LKB Ultroscan) equipped with a peak integrator (Hewlett Packard 3390). A standard curve for KRP staining was generated by analyzing the staining intensity of dilutions from a stock KRP solution, the concentration of which was determined by amino acid analysis. The determination of KRP:myosin molar ratios in the differential centrifugation binding assays was determined by densitometric analysis of Coomassie Blue-stained gels containing the various supernatant and pellet fractions. Myosin content was assessed by scans of the MLC-17 band. The relative staining intensity of MLC-17 and KRP and molecular weights of 16,800 and 17,200, respectively, were used to determine the relative concentrations of MLC-17 and KRP in SDS-PAGE analyses.

Electron Microscopy—Unphosphorylated gizzard myosin (3–5 μM) that had been dialyzed against buffer A was depolymerized by adding ATP to 1 mM. Following a 5-min incubation at 20 °C, it was centrifuged at 85000 × *g* for 30 min at 20 °C to remove residual filaments (with fresh myosin, less than 10% of the total myosin was found in the pellet). KRP was added to the supernatant, and, following a 10-min incubation at 20 °C, the mixture was diluted with ice-cold buffer A containing ATP to give a final protein concentration of 50 μg/ml. The samples were then immediately applied to UV-irradiated Formar-carbon grids and stained with uranyl acetate as described by Trybus and Lowey (1987). Electron microscopy was performed on a JEM 100CX electron microscope operated at 100 kV. Magnification was calibrated using the catalase crystal repeat.

Quantitation of KRP and MLCK in Chicken Gizzard—Frozen chicken gizzard was placed in liquid nitrogen and pulverized using a mortar and pestle. Hot 4 ×X SDS sample buffer supplemented with 1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride was added to the powder after the nitrogen evaporated. The suspended sample was boiled for 5 min and homogenized by Polytron for 1 min at maximal setting. The sample was clarified by centrifugation in a Microfuge for 5 min, and the supernatant was subjected to electrophoresis on 12.5% acrylamide minislab gels. Included on these gels were standards of purified chicken gizzard KRP and MLCK for quantitation purposes. Because MLCK and KRP behave very differently in electrotransfer, the gel was cut along the horizontal axis and the upper and lower parts transferred to nitrocellulose sheets (Schleicher & Schuell 0.45 μm). The upper part containing MLCK was transferred at 80V for 4 h. The lower part containing KRP was processed by the method of Van Eldik and Wolchok (1984). Briefly, the transfer was for 1.5 h in Tris-glycine/methanol transfer buffer with subsequent treatment with 0.2% glutaraldehyde in phosphate-buffered saline for 45 min at room temperature. The blots were then processed as described (Van Eldik and Wolchok, 1984; Belkin *et al.*, 1988). MLCK and KRP were detected with rabbit anti-MLCK antibody 859, which has been shown (Collinge *et al.*, 1992) to detect both MLCK and KRP. A ^{125}I-labeled goat anti-rabbit antibody was used to quantitate the immunoreactive proteins. Proteins were quantitated by excising corresponding bands from the nitrocellulose filters and counting in a γ counter, and by densitometry of autoradiograms of the blots exposed to x-ray film for 12 h. Both methods gave similar results. The KRP:MLCK molar ratio was estimated from calibration curves of amount of radioactivity in the bands corresponding to KRP or MLCK *versus* the amount of KRP or MLCK loaded on the gel. The amount of KRP or MLCK in the samples was then determined from the radioactivity in each of

the triplicate lanes of each dilution of tissue extract. The preparation and analysis of KRP and MLCK mRNAs were done exactly as previously described (Collinge *et al.*, 1992).

In Vitro Motility and Actin-activated ATPase Assays—Motility assays were performed as previously described (Homsher *et al.*, 1992). The assay buffer contained 20 mM KCl, 20 mM MOPS (pH 7.2), 5 mM $MgCl_2$, 0.1 mM EGTA, 10 mM DTT, 0.7% methyl cellulose, 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 30 °C.

The actin-activated MgATPase activity of unphosphorylated gizzard myosin was measured in a buffer containing 50 mM KCl, 10 mM $MgCl_2$, 20 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM [^{32}P]ATP, 1 mM DTT, 0.01 mM actin, 0.0005 mM myosin, and either 0 or 0.010 mM KRP at 37 °C. Five time points were taken to ensure linearity of the reaction. The release of radioactive phosphate was measured by the method of Pollard and Korn (1973).

RESULTS AND DISCUSSION

Myosin Binding Activity of KRP—KRP binding to myosin could be demonstrated by the use of a standard sedimentation assay (Fig. 1). The presence of a KRP band in the pellet fraction is indicative of KRP binding to myosin. KRP binds to unphosphorylated turkey gizzard myosin (Fig. 1, *panel 1*), but poorly to phosphorylated gizzard myosin (Fig. 1, *panel 2*). Little interaction, if any, was detected between KRP and skeletal muscle myosin (Fig. 1, *panel 3*). KRP still showed myosin binding activity in the presence of 100 mM NaCl and after heat treatment conditions (5 min at 95 °C). As summarized in Table I, as long as KRP is present in the incubation mixture in at least 2-fold molar excess over myosin, the molar ratio of KRP to myosin in the pellet (*i.e.* associated with myosin) is at least unity. These results are consistent with the apparent affinity of KRP for myosin being in the micromolar range under these conditions, and with the KRP-myosin complexes being in equilibrium with the free proteins. When CaM, an acidic protein with similar physicochemical features, was substituted for KRP, no interaction of CaM with myosin was observed. Using a sedimentation assay, we also failed to detect (see below) any interaction of KRP with F-actin in the presence or absence of actin-binding proteins tropomyosin, caldesmon and calponin. Thus, the interaction of KRP with unphosphorylated smooth muscle myosin appears to be a selective one that occurs under conditions of physiological ionic strength and pH.

The KRP Domain of MLCK and Binding to Myosin and Actin—The myosin binding activity associated with KRP suggested that MLCK might use its KRP domain (Shoemaker *et al.*, 1990) to interact with myosin. In order to assess this hypothesis, we used the procedure of Sellers and Pato (1984) to test the ability of the MLCK construct, rMLCK1 (Shoemaker *et al.*, 1990), which lacks most of the KRP region but has CaM-dependent kinase activity, to co-sediment with myosin. As shown in Fig. 2*A*, rMLCK1 is diminished in its ability

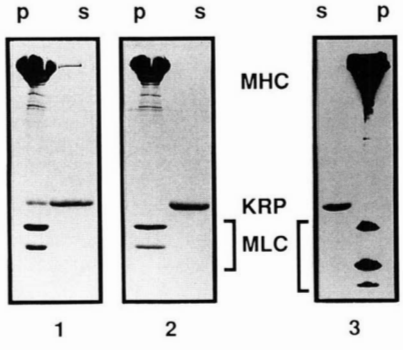

FIG. 1. **Binding of KRP to different myosins.** Each panel shows the SDS-PAGE analysis of the pellet (*P*) and the supernatant (*S*) of KRP-myosin mixtures after sedimentation at 85,000 × *g*. *Panel 1*, 4 μM unphosphorylated turkey gizzard myosin + 12 μM chicken gizzard KRP; *panel 2*, 4 μM phosphorylated turkey gizzard myosin + 12 μM chicken gizzard KRP; *panel 3*, 4 μM unphosphorylated rabbit skeletal myosin + 9 μM chicken gizzard KRP. The samples with skeletal muscle myosin were run on 12.5% (w/v) gel containing twice the *N,N*-methylene-bisacrylamide concentration as standard gels to effect the separation of KRP and light chain bands. *MHC*, myosin heavy chain; *MLC*, myosin light chains.

TABLE I

Stoichiometry of KRP and myosin in sedimentable myosin complexes

Amount of protein added to incubation mixture		Ratio in pellet (KRP/myosin)[b]
Myosin[a]	KRP	
μM	μM	mol:mol
3	6	1.2
3	18	1.2
4	11	1.1
5	4	0.9
5	13	1.3
5	20	1.4

[a] Unphosphorylated gizzard myosin.

[b] The quantitation was done by gel densitometry of KRP and MLC-17 as described under "Materials and Methods."

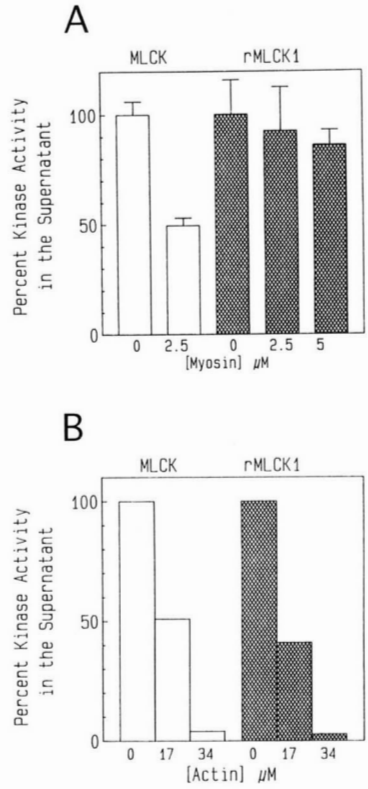

FIG. 2. **Differences in the interaction of myosin with MLCK and rMLCK1, a truncated MLCK analog lacking most of the KRP domain.** *A*, chicken gizzard MLCK (0.05 μM) or rMLCK1 (0.05 μM), an MLCK mutant lacking a KRP region (Shoemaker *et al.*, 1990), were incubated in the presence of turkey gizzard myosin (2.5 or 5 μM), and the kinase activity of MLCK (*open bars*) and rMLCK1 (*cross-hatched bars*) was measured after sedimentation. The activity remaining in the supernatant is expressed as a percentage of the total found in the absence of myosin. Each *bar* represents the mean ± S.E. of quadruplicate determinations. *B*, as a control, the ability of chicken gizzard MLCK (0.05 μM) or rMLCK1 (0.05 μM) to interact with rabbit skeletal muscle actin was tested by the procedure of Sellers and Pato (1984). In two separate experiments no detectable differences were found between the MLCK with or without an intact KRP domain. The histograms show the average of duplicate determinations in one of the two assays.

to bind to unphosphorylated myosin compared to MLCK, but rMLCK1 retains its ability to associate with actin like native MLCK (Fig. 2*B*). In addition, we observed a competition between KRP and MLCK for myosin. As summarized in Table II, increasing the KRP concentration in a mixture containing myosin and MLCK increases the amount of MLCK found in the supernatant. The results of these experiments are consistent with the KRP amino acid sequence also having myosin binding activity when it is found as a domain of MLCK.

Although KRP does not have a major effect on the CaM-dependent phosphotranseferase activity of MLCK with peptide or light chain substrates (Shoemaker *et al.*, 1990; Collinge *et al.*, 1992), the competition between KRP and MLCK for myosin binding suggested that KRP might indirectly affect the *in vitro* phosphorylation of myosin light chains when smooth muscle HMM is used as a substrate. As indicated by the data in Fig. 3, KRP attenuates the initial rate of light chain phosphorylation by MLCK when HMM is used as a substrate. While these results raise the interesting possibility that the same genetic locus might encode a protein kinase and an inhibitor of its action, it should be noted that the maximal effect on the rate (not extent) of phosphorylation is only 40% of control values (Fig. 3). These data (Fig. 3 and Table II) and previous reports (Shoemaker *et al.*, 1990; Collinge *et al.*, 1992) are consistent with a model in which this inhibitory effect of KRP is due to a decrease in MLCK binding to myosin. The *in vivo* significance of the competition between KRP and MLCK for myosin binding and its resultant diminution of the initial rate of light chain phosphorylation is not known and cannot be deduced from the results of this study alone.

In terms of the structural basis of recognition and potential importance in subcellular targeting of MLCK, the results are consistent with both the catalytic and KRP domains of MLCK being important in interactions with myosin filaments. The catalytic domain could contribute to binding due to direct interactions with the light chain substrate, and the KRP domain could contribute to myosin binding through interactions distinct from, but related to, light chain interactions. The features of myosin that are important for KRP binding, described in a subsequent section, are also consistent with this hypothesis.

KRP and Myosin Filament Stabilization—In a search of the consequences of KRP binding to unphosphorylated smooth muscle myosin, we observed that KRP shifts, in a concentration-dependent manner, the equilibrium between monomeric and filamentous myosin in the presence of 1 mM MgATP. As shown in Table III, either at 50 or 100 mM NaCl, 1 mM MgATP readily dissolves unphosphorylated myosin filaments so that the myosin is no longer sedimentable. However, increasing the amount of KRP results in a recovery of more myosin in the pellet, indicative of KRP enhancement of myosin association in the presence of ATP.

The myosin structures formed in the presence and absence of ATP were studied by electron microscopy (EM). As shown in Fig. 4*A*, myosin in the presence of ATP but the absence of KRP is in a non-filamentous state. As shown in Fig. 4*B*, multiple myosin minifilaments are observed in the field if KRP is present. The minifilaments formed in the presence of KRP look similar to those observed for synthetic smooth muscle myosin filaments in the absence of ATP (Sobieszek and Small, 1972; Trybus and Lowey, 1987) and native thick filaments released from smooth muscle cells (Small, 1977).

The results of sedimentation and EM studies agree with each other and demonstrate that the interaction of KRP with unphosphorylated myosin is a selective one that promotes the *in vitro* assembly of unphosphorylated myosin filaments that are relatively resistant to ATP depolymerization. The ability of KRP to protect or promote myosin filament structures is reminiscent of that seen as a result of LC20 phosphorylation by MLCK. However, in contrast to MLCK, KRP does not contain protein kinase catalytic motifs in its amino acid sequence, and KRP preparations lack protein kinase activity (Shoemaker *et al.*, 1990; Collinge *et al.*, 1992). Furthermore, a variety of control experiments exclude the possibility that the activity of KRP is due to kinase contamination of KRP preparations. For example, in addition to the failure of KRP preparations to catalyze the transfer of isotopic phosphate from ATP to smooth muscle myosin, KRP preparations do

TABLE II

Effect of KRP on the co-sedimentation of MLCK and myosin

The turkey gizzard myosin suspension (5 μM) was mixed with 3 μM chicken gizzard MLCK and KRP in buffer A, then centrifuged and analyzed by SDS-PAGE as described under "Materials and Methods." The percentage of MLCK in the supernatant was quantified from scanning densitometry.

KRP added	MLCK in supernatant
μM	*% of amount added*
No addition	12
4	43
28	95

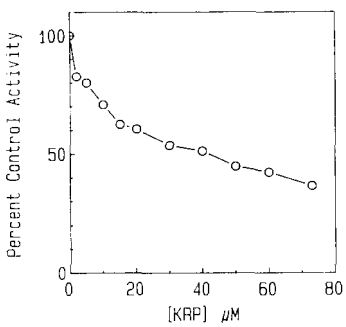

FIG. 3. **Effect of KRP on initial rate of light chain phosphorylation using HMM as substrate.** HMM (10 μM) was phosphorylated by MLCK as described under "Materials and Methods" in the presence of increasing concentrations of KRP. The rate of phosphorylation is expressed as a percent of the control which did not contain KRP. The control specific activity in the absence of KRP was 3.7 μmol/min/mg. Each *point* represents the mean of triplicate experiments.

TABLE III

Effect of KRP on myosin sedimentation in the presence of ATP

The turkey gizzard myosin suspension (5 μM) in buffer A containing either 50 or 100 mM NaCl was mixed with ATP at a final concentration of 1 mM and processed as described under "Materials and Methods." After a 10-min incubation at 20 °C, chicken gizzard KRP was added and the mixture incubated for 20 min at 20 °C. Following centrifugation and SDS-PAGE, the percentage of myosin in the pellet and supernatant was quantified from scanning densitometry as described under "Materials and Methods," where 100% = the amount of myosin detected in the supernatant in the presence of ATP alone.

KRP added	% Myosin found in	
	Pellet	Supernatant
μM		
No addition	0	100
2.5	15	85
10	30	70
25	50	50

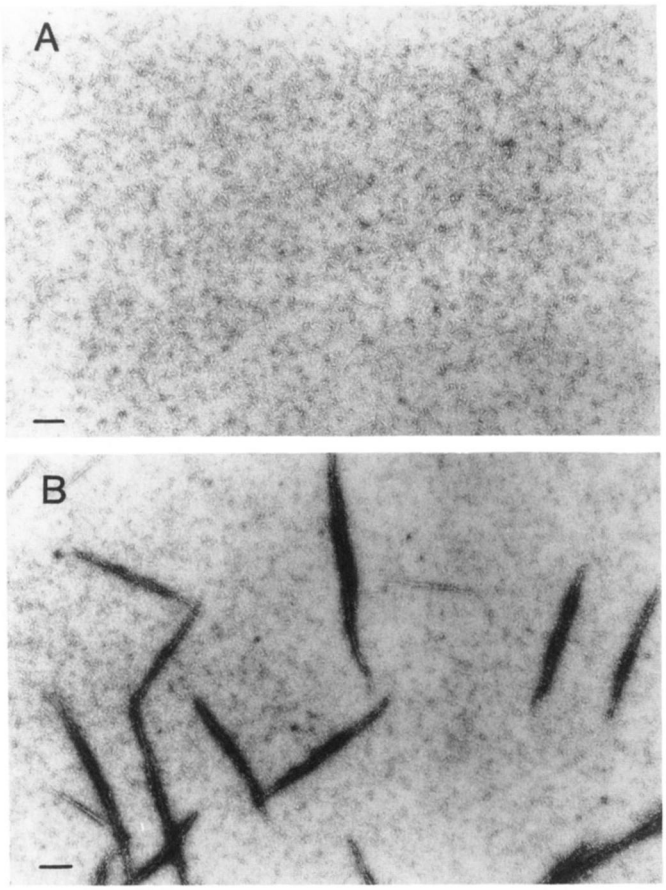

FIG. 4. **Electron microscopy of myosin filaments formed in the presence of ATP and KRP.** Gizzard myosin depolymerized by the addition of 1 mM ATP (*panel A*) reassembled into minifilaments in the presence of gizzard KRP (*panel B*). The ratio of KRP:myosin in the incubation mixture was 10:1. The *scale bar* in each panel represents 0.1 μm.

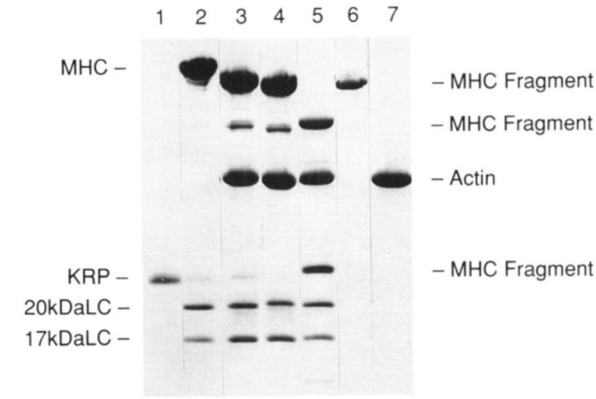

FIG. 5. **KRP binding to myosin fragments.** The interaction of KRP with myosin fragments was assayed by a standard sedimentation assay, followed by SDS-PAGE analysis of the resultant supernatant and pellet fractions, as described under "Materials and Methods." *Lane 1* is the purified KRP standard, and *lanes 2–7* are the pellet fractions following sedimentation of KRP (5 μM) with: myosin (*lane 2*), HMM (*lane 3*), phosphorylated HMM (*lane 4*), S1 (*lane 5*), myosin rod (*lane 6*), or actin (*lane 7*).

not stimulate the actin-activated MgATPase activity of myosin and fail to support actin filament movement over unphosphorylated smooth muscle myosin in an *in vitro* motility assay (data not shown). The results indicate that the effect of KRP on myosin polymerization is probably through a stoichiometric protein binding mechanism rather than enzymatic modification. Hence, the effect of KRP on myosin can be distinguished functionally from that of MLCK. Studies in progress are aimed to elucidate whether the myosin filaments promoted by KRP *versus* phosphorylation by MLCK have structural differences as well.

KRP and the Regulation of Unphosphorylated Myosin Conformation—One working hypothesis based on the experimental results proposes that KRP alters the conformation of myosin in such a way that it cannot as readily adopt a folded conformation, thereby favoring filament formation. This assumes that the KRP binding occurs near a region of the myosin molecule important for conformational changes. The demonstration that phosphorylation of myosin LC20 by MLCK decreases the affinity of myosin for KRP (Fig. 1), and that KRP affects the phosphorylation of HMM by MLCK (Fig. 3), suggested that KRP may bind near the LC20 binding site. In order to gain further insight into which regions of myosin are important for interactions with KRP, the binding of KRP to various subfragments of myosin was studied using sedimentation assays. Actin was used to sediment the soluble myosin subfragments HMM and S1. There was no binding of KRP to actin (Fig. 5, *lane 7*). KRP bound to unphosphorylated HMM (Fig. 5, *lane 3*) but not to phosphorylated HMM (Fig. 5, *lane 4*), similar to results obtained with the whole myosin molecule. In contrast, KRP does not bind to S1, even though it retains both light chains (Fig. 5, *lane 5*), nor does it bind to the myosin rod fragment (Fig. 5, *lane 6*) under these experimental conditions. These results suggest that the presence of both S2 and the regulatory light chain (LC_{20}) are required for KRP binding to myosin because both S1 containing LC_{20}, and the myosin rod, which has an S2 portion, failed to bind KRP. Additional experiments revealed that purified S2 failed to compete with HMM for KRP binding, again suggesting that the binding site is a more extended structure perhaps involving both chains of the myosin molecule. These data are consistent with the myosin binding data in Table I, which demonstrate that one KRP molecule binds per myosin molecule. In this regard, it is interesting to speculate that KRP may bind to the "neck" region on myosin where the two LC_{20}s and the heavy chains are associated near the beginning of the coiled-coil portion of the myosin rod. If this were the case, KRP could suppress the formation of the folded 10 S conformation of myosin by directly competing with the myosin tail for binding to this portion of the head, and suppression of the 10 S conformation would lead to stabilization of myosin filaments.

Abundance of KRP in Gizzard Tissue—The features of KRP revealed in this study suggest that it might influence the dynamics of thick filaments in smooth muscle if KRP is as abundant as myosin in this tissue. Previous studies (Collinge *et al.*, 1992) have shown that the chicken gizzard 2.7-kb mRNA that encodes KRP is at least 10-fold more abundant than the 5.5-kb mRNA that encodes MLCK. The Northern blot in Fig. 6 readily reveals that the KRP mRNA is more abundant than the MLCK mRNA. The probe used in Fig. 6 includes the 3′-untranslated portion of the two mRNAs, thereby biasing the detection toward the noncoding part of the mRNAs. This maximizes the specificity of the probe and makes it less probable that mRNAs coding for related proteins from other genes would interfere with the analysis. Consistent with the greater abundance of KRP mRNA, quantitative Western blot analyses of tissue extracts (see "Materials and Methods") revealed a KRP:MLCK molar ratio of 18:1. Based on the concentration of MLCK in gizzard tissue of approximately 1.6–4.6 μM (Adelstein and Klee, 1982; Ngai and Walsh, 1985), the KRP concentration is approximately 80–90 μM.

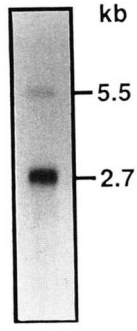

FIG. 6. **Northern blot analysis of KRP and MLCK mRNAs in adult chicken gizzard tissue.** Chicken gizzard poly(A)$^+$ RNA (9 μg) was separated on a 1% (w/v) agarose/formaldehyde gel, and transferred to Hybond-N nylon membrane (Amersham Corp.) as previously described (Collinge *et al.*, 1992). The blot was hybridized with a probe located in the 3′-noncoding region that is common to both mRNAs: nucleotides 4166–4404 of Collinge *et al.* (1992). The 2.7-kb KRP mRNA and the 5.5-kb MLCK mRNA are indicated. *Bst*EII-digested λ phage DNA were used as DNA size standards (not shown).

This is within the range of the myosin concentration, which has been estimated at 80 μM (Ruegg, 1986). Thus, the relative amount of KRP in gizzard tissue is sufficient for it to function as a component of the thick filament, thereby providing a mechanism for retention of thick filament integrity in relaxed smooth muscle.

Clearly, the results of additional more detailed studies, which are in progress, are required before any firm conclusions can be drawn concerning the *in vivo* role of KRP. However, the results presented here provide the first description of a biochemical function for KRP, one of the products of a novel genetic locus (Collinge *et al.*, 1992), and a foundation for new avenues of investigation in smooth muscle biology.

Acknowledgments—We thank Drs. Linda Van Eldik and Jack Wells for stimulating discussions and critical advice and Dr. Joel Hardman for advice and encouragement.

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